

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Albrecht t al.)	
)	
Serial No.:	09/748,642)	Examiner: J. Shultz
)	
Filed:	December 22, 2000)	
)	Art Unit: 1635
For:	INHIBITION OF CELLULAR)	
	PROTEASES)	
)	

**DECLARATION OF THOMAS ALBRECHT AND ZHENPING CHEN
UNDER 37 C.F.R. ' 1.132**

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

We, THOMAS ALBRECHT AND ZHENPING CHEN, declare:

1. I, Thomas Albrecht received a Ph.D. in 1973 from the Pennsylvania State University College of Medicine in the fields of Microbiology and Biochemistry.

2. From 1986 to this date, I was employed as a Professor at The University of Texas Medical Branch.

3. I, Zhenping Chen received a M.D. in 1982 from the Zhongshan Medical College (which is now called Zhongshan University) and an M.S. in 1988 from Sun Yat-sen University of Medical Sciences (which is now called Zhongshan University) in the field of Oncology.

4. From 1997 to 2006, I was employed as a post-doctoral research associate at the Dept. of Microbiology and Immunology, the University of Texas Medical Branch.

5. I am currently employed as a post-doctoral fellow in the Dept. of Otolaryngology, the University of Texas Medical Branch.

6. We are the inventors of the subject matter claimed in the above-identified patent application.

7. In the above-identified patent application, in an Office Action dated October 5, 2006, at page 2-3, the U.S. Patent and Trademark Office (PTO) rejected claims 6, 7, 14 and 15 under 35 U.S.C. '112(first paragraph) for lack of enablement because:

[t]he specification as filed does not provide any guidance or examples that would enable a skilled artisan to use the disclosed compounds or methods of using said compounds in vivo, since the prior art is silent on the use of such inhibitors in vivo.

The comments set forth below are intended to demonstrate that calpain inhibitors E64D and Z-Leu-Leu-H are cell permeable and, thus, able to be used in *in vivo* methods.

8. As shown in a paper co-authored by the undersigned inventors, Chen et al., A Degradation of p21^{Cip1} in Cells Productively Infected with Human Cytomegalovirus®, J. Virol. 75(8):3613-625(2001)(attached hereto), calpain inhibitors E63d and Z-Leu-Leu-H are cell permeable. (See Page 3617, first paragraph).

9. Further, online catalogs carrying calpain inhibitors E64d and Z-Leu-Leu-H, such as Calbiochem and Peptides International (print out of relevant pages attached hereto) indicate that these calpain inhibitors are cell permeable.

10. Further, as shown in Swapan et al., A Cell Death in Spinal Cord Injury (SCI) Requires de Novo Protein Synthesis®, Annals N.Y. Academy of Sciences 939:436-49 (2001)(abstract), calpain inhibitors, such as E64d, have been used in animal models to treat spinal cord injuries. Results indicate that calpain

activity was almost undetectable in the E64d treated rats.


11. In addition, in the Office Action dated October dated October 5, 2006, at page 3-4, the PTO rejected claims 6 and 7 under 35 U.S.C. '102(a) as anticipated by Chen et al., Proceedings of the American Association for Cancer Research Annual Meeting, 40:447-48(1999) (AChen@). However, Chen describes the work of the inventors.

12. Listed co-author E. Knutson was a technician working in the lab of the inventors and performed experiments under the supervision, direction and control of the inventors.


13. Listed co-author S. Liu was a post-doc fellow working in the lab of the inventors and performed experiments under the supervision, direction and control of the inventors.

14. Listed co-author A. Kurosky was a Professor in the Department of Human Biological Chemistry and Genetics at the University of Texas Medical Branch. Kurosky was consulted to review the findings of the inventors, such findings which were under the direction and control of the inventors

15. We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

5 March 2007 
Date

Thomas Albrecht

5 March 2007 
Date

Zhenping Chen

Degradation of p21^{cp1} in Cells Productively Infected with Human Cytomegalovirus

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Human cytomegalovirus (HCMV) stimulates arrested cells to enter the cell cycle by activating cyclin-dependent kinases (Cdk), notably Cdk2. Several mechanisms are involved in the activation of Cdk2 from the causes a substantial increase in the abundance of cyclin E and stimulates translocation of Cdk2 from the cytoplasm to the nucleus. Further, the abundance of the Cdk inhibitors (CKIs) p21^{cp1/waf1} (p21^{cp1}) and p27^{kip1} is substantially reduced. The activity of cyclin E/Cdk2 increases as levels of CKIs, particularly p21^{cp1}, fall. We have previously shown that these phenomena contribute to priming the cell for efficient replication of HCMV. In this study, the mechanisms responsible for the decrease in p21^{cp1} levels after HCMV infection were investigated by measuring p21^{cp1} RNA and protein levels in permissive human lung (LU) fibroblasts after HCMV infection. Northern blot analysis revealed that p21^{cp1} RNA levels increased briefly at 3 h after HCMV infection and then decreased to their nadir at 24 h; thereafter, RNA levels increased to about 60% of the preinfection level. Western blot analysis demonstrated that the relative abundance of p21^{cp1} protein roughly paralleled the observed changes in initial RNA levels; however, the final levels of protein were much lower than preinfection levels. After a transient increase at 3 h postinfection, p21^{cp1} abundance declined sharply over the next 24 h and remained at a very low level through 96 h postinfection. The disparity between p21^{cp1} RNA and protein levels suggested that the degradation of p21^{cp1} might be affected in HCMV-infected cells. Treatment of HCMV-infected cells with MG132, an inhibitor of proteasome-mediated proteolysis, provided substantial protection of p21^{cp1} in mock-infected cells, but MG132 was much less effective in protecting p21^{cp1} in HCMV-infected cells. The addition of E64d or Z-Leu-Leu-H, each an inhibitor of calpain activity, to HCMV-infected cells substantially increased the abundance of p21^{cp1} in a concentration-dependent manner. To verify that p21^{cp1} was a substrate for calpain, purified recombinant p21^{cp1} was incubated with either m-calpain or μ -calpain, which resulted in rapid proteolysis of p21^{cp1}. E64d inhibited the proteolysis of p21^{cp1} catalyzed by either m-calpain or μ -calpain. Direct measurement of calpain activity in HCMV-infected cells indicated that HCMV infection induced a substantial and sustained increase in calpain activity, although there was no change in the abundance of either m- or μ -calpain or the endogenous calpain inhibitor calpastatin. The observed increase of calpain activity was consistent with the increases in intracellular free Ca²⁺ and phospholipid degradation in HCMV-infected LU cells reported previously from our laboratory. Considered together, these results suggest that the increase in calpain activity observed following HCMV infection contributes significantly to the reduction of p21^{cp1} levels and the resultant cell cycle progression.

Human cytomegalovirus (HCMV) infection is widespread among human populations, primarily as a subclinical persistent infection. Furthermore, HCMV infection is a major cause of morbidity and mortality in several well-studied risk groups. These include congenitally infected infants and individuals with compromised immune systems, particularly after human immunodeficiency virus infection or immunosuppressive therapy for tissue transplantation (for reviews, see references 8, 29, 68, and 72). The clinical management of these infections is still problematic. Although several agents with potent antiviral activity for HCMV infection both *in vitro* and *in vivo* have been identified, the toxicity associated with the long-term use of these drugs makes clinical management difficult, and drug-resistant strains of HCMV have emerged (for a review, see reference 61). Thus, there continues to be great interest in improving our understanding of the replication of HCMV with

a view toward developing more effective approaches to control these infections.

HCMV replication is associated with extensive modifications of cellular metabolism (reviewed in references 4 and 5), leading to a number of physiologic changes and the activation of a large number of cellular genes (91). Initially, HCMV infection induces a series of cellular responses that resemble the immediate-early events observed following activation of serum-arrested cells by serum growth factors (4). These include hydrolysis of phosphatidylinositol 4,5-bisphosphate, yielding increased cellular levels of *sn*-1,2-diacylglycerol (DG) and inositol 1,4,5-trisphosphate (81); increased release of arachidonic acid and its metabolites (1, 2); changes in Ca²⁺ homeostasis, including Ca²⁺ influx, release of Ca²⁺ stores, and an increase in intracellular free Ca²⁺ (58); transcriptional activation of cellular oncogenes *c-fos*, *c-jun*, and *c-myc* (11, 12, 13); and increased activity of the DNA-binding proteins NF κ B, AP-1, and CREB (14). The signaling cascade induced by HCMV infection induces a robust mitogenic response, as evidenced by the ability of HCMV to stimulate cell cycle entry by density-arrested cells, which are resistant to stimulation by serum

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growth factors (19). Recent results indicate that productive HCMV infection stimulates cell cycle progression in either serum- or density-arrested cells through late G₁ phase to a point at or near the G₁/S boundary (19, 30, 52). Closely associated with this limited traverse of the cell cycle is an increase in cyclin E/cyclin-dependent kinase 2 (Cdk2) activity, hereafter referred to as E kinase activity, and hyperphosphorylation of pRb, releasing E2F (45). Activation of E2F, together with Myc, leads to an increase in the cellular levels of a large number of genes involved in nucleotide biosynthesis which primes the infected cell for DNA synthesis (5, 7).

Three HCMV-induced events appear to be necessary for activation of E kinase activity: (i) transcriptional activation of cyclin E (17), (ii) translocation of Cdk2 from the cytoplasm to the nucleus (20), and (iii) a substantial decrease in the nuclear levels of the cyclin kinase inhibitors (CKIs) p21^{cip1}/waf1 (hereafter p21^{cip1}) and p27^{kip1} (19). Activation of E kinase appears to be critical for efficient HCMV replication, since drugs that interfere with the activity of Cdk2 substantially reduce infectious yields (18). The precise mechanisms through which these virus-induced cellular modifications are achieved are poorly understood. Our long-term goal is to better understand the events that prime the cell for HCMV replication. We are especially interested in the mechanism of reduction of cellular levels of p21^{cip1} during HCMV infection, since this event appears to be closely associated with the increase in E kinase activity (19).

p21^{cip1} is a potent inhibitor of Cdks (41, 42, 84) and is a critical p53-induced downstream effector in the growth-suppressive pathway (33). p21^{cip1} binds cyclin-Cdk complexes, thereby inhibiting the activity of Cdks, such as Cdk2, Cdk3, Cdk4, and Cdk6, and consequently inhibiting cell cycle progression. In addition, p21^{cip1} interacts with proliferating cell nuclear antigen (36) and gadd45 (48), affecting their function, e.g., interfering with DNA replication and repair (24, 36, 51, 60, 62, 82, 86). p21^{cip1} may also be involved in p53-mediated apoptosis (39) and in the control of cell senescence (57).

Despite significant advances in our understanding of how p21^{cip1} exerts its biological effects and is transcriptionally regulated, little is known about how the stability of p21^{cip1} is regulated under the physiologic conditions associated with disease and other forms of stress. Nucleosomal cytoplasmic protease systems have been identified as important regulators of cell cycle progression (26, 28, 35, 59, 70, 71). Two prominent cytoplasmic protease pathways have been identified: the ubiquitin-proteasome pathway and the calpain pathway. Many of the cell cycle regulatory proteins that are degraded at specific points in the cell cycle, e.g., cyclins A, B, and E, are substrates of the ubiquitin-proteasome pathway. It has also been reported that p21^{cip1} is subject to proteolysis by ubiquitin-mediated proteasome degradation (10, 35, 38, 85). In this study, we undertook a series of experiments to investigate the proteolytic degradation of p21^{cip1} in cells productively infected with HCMV. Consistent with previous work, we observed that the ubiquitin-proteasome pathway is the predominant mechanism mediating the proteolysis of p21^{cip1} in mock-infected or noninfected human embryonic lung (LU) cells. In HCMV-infected LU cells, calpain is activated and is the predominant proteolytic mechanism mediating degradation of p21^{cip1}. HCMV infection induced a substantial and prolonged increase in calpain activity

when measured in intact cells using fluorogenic substrates or in lysates of infected cells by zymography. The time course for the HCMV-induced increase in calpain activity was consistent with the temporal pattern of p21^{cip1} degradation, and inhibitors of calpain-mediated proteolysis interfered with its abundance. These findings provide an explanation for the sustained decrease in p21^{cip1} abundance observed in HCMV-infected cells and, accordingly, help to explain the prolonged activation of E kinase in HCMV infection.

MATERIALS AND METHODS

Cell culture and growth arrest. LU cells (3) were propagated in Eagle's minimum essential medium containing 10% fetal bovine serum (FBS) and penicillin (100 U/ml)-streptomycin (100 µg/ml) in a 5% CO₂ in air atmosphere. The cells were density arrested as described previously in detail (19).

Virus stocks and productive infection. The AD169 strain of HCMV was propagated in LU cells as previously described in detail (19). Infectivities of virus stocks, determined by plaque assay (3), typically were between 8.0 × 10⁶ and 4.0 × 10⁷ PFU/ml. LU cells were infected with HCMV at a multiplicity of 5 PFU/cell to provide a uniform infection as described in detail by Breesma et al. (19). Virus stocks and cell cultures were routinely examined for mycoplasma and were free from detectable contamination.

RNA isolation. RNA was extracted using Tri Reagent (Molecular Research Center, Inc., Cincinnati, Ohio), which contains phenol and guanidine thiocyanate, according to the manufacturer's protocol (25).

Preparation of probes. A 32P-labeled DNA probe was prepared from plasmid pC-waf-1S (generously provided by B. Vogelstein [33]), which harbors a p21^{cip1} cDNA. The probe was prepared by digesting the plasmid with EcoRI for 18S. A probe derived from plasmid pSb (16), which contains the cDNA for 18S rRNA, was used to ensure uniform loading of gels. Plasmids were introduced into competent *Escherichia coli* DH5α cells and amplified. DNA fragments comprising p21^{cip1} and 18S cDNA were excised from isolated plasmid DNA by using the restriction endonucleases NotI and BamHI-EcoRI, respectively. The probes were labeled using the Megaprime DNA Labelling System (Amersham Pharmacia Biotech, Piscataway, N.J.).

Electrophoresis and Northern hybridization. Northern hybridization was performed as described previously (17). Total cellular RNA (20 µg/lane) was evaluated under denaturing conditions in formaldehyde-agarose gels, containing 1% agarose, 20 mM 3-(N-morpholino) propanesulfonic acid, 1 mM EDTA, 8 mM sodium acetate, and 2.2 M formaldehyde. After separation, the RNA was transferred to MS1 nylon membranes (Miles Separations, Inc., Woburn, Mass.) for 18 h. The RNA was prehybridized in Rapid-Hyb buffer (Amersham Pharmacia Biotech, Arlington Heights, Ill.) containing denatured salmon sperm DNA (100 µg/ml) at 65°C for 1 h. 32P-labeled p21^{cip1} or 18S probe was added and hybridized for 2 h at 65°C. Membranes were washed twice with 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA [pH 7.7])–0.1% sodium dodecyl sulfate (SDS) for 15 min at 65°C, once with 1× SSPE–0.1% SDS at 42°C, and twice with 0.1× SSPE–0.1% SDS at 42°C. The probe remaining on the filter was detected by autoradiography (Kodak [Rochester, N.Y.] X-Omat film for 1 to 16 h at –80°C).

Western blots. Polyclonal and monoclonal antibodies were provided by or obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). Antibodies against μ -calpain, ν -calpain, and calpastatin were generously provided by R. L. Mellgren (88). Cells were harvested as we described previously (19) by dislodging the cells with a cell lifter in phosphate-buffered saline (50 mM Tris-HCl [pH 7.4] containing 150 mM NaCl and 0.5% NP-40, with 1 mM dithiothreitol [DTT], 1 mM NaVO₃, 50 mM NaF, and protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 25 µg of trypsin inhibitor/ml, 25 µg of aprotinin/ml, 1 M benzamide, and 25 µg of pepstatin A/ml] added just before use). Cellular debris was removed by sedimentation, and the supernatant fluids were reserved. The protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, Ill.). Whole-cell extracts (40 µg/lane) were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE), and the polyphosphates were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, Cal.) as described previously (19). Antigen-antibody reactions were detected by the enhanced chemiluminescence assay (Amersham Pharmacia Biotech) as recommended by the manufacturer.

Measurement of intracellular calpain activity. Calpain activity was measured using the cell-permeant fluorogenic calpain substrate *tert*-butylcarboxy-DTT.

Image processing. All experiments were repeated at least twice, and either data from a representative experiment or means of data from two or more experiments were selected for presentation. Chemiluminescent samples were exposed for intervals that ensured a linear response, as determined by standardization. All radiographic films were analyzed using an AlphaImager 2000 documentation and analysis system (Alpha Innotech Corp.) and the manufacturer's software (AlphaImager 2000, version 3.x). The images were quantified and recorded as tagged image format files, which were then used to prepare the graphic images presented in this report.

Differential effects of an inhibitor of the ubiquitin-proteasome proteolytic pathway on p21^{cp1} degradation during HCMV infection and mock infection. Since the ubiquitin-proteasome pathway is reported to be responsible for degrading many of the cell cycle regulatory proteins, we investigated whether or not the decrease of p21^{cp1} in HCMV-infected cells was due to a proteasome-mediated mechanism. HCMV (PFU/cell)-infected and mock-infected LU cells were treated with MG132, an inhibitor of proteasome-mediated proteolysis (56, 69), at 1 h p.i., and p21^{cp1} abundance was evaluated at 24 h p.i. The results of a representative experiment are shown in Fig. 2A. By 24 h p.i., p21^{cp1} levels were substantially lower in HCMV-infected LU cells (Fig. 2A) than in the control (mock-infected cells). MG132 stabilized p21^{cp1} in mock-infected, density-arrested LU cells but had only a limited effect on stabilization of p21^{cp1} in HCMV-infected cells (Fig. 2). Mock-infected, density-arrested LU cells treated with MG132 (0 to 10 μ M) demonstrated a concentration-dependent increase in the abundance of p21^{cp1}. A greater than fivefold increase in p21^{cp1} abundance relative to either the dimethyl sulfoxide (DMSO) control (lane 2) or cells in the absence of any chemical (lane 1) was observed at concentrations of 5 and 10 μ M (Fig. 2). In HCMV-infected cells, smaller increases in p21^{cp1} abundance were observed at MG132 concentrations from 2.5 to 10 μ M, reaching a maximum 2.5-fold increase at 10 μ M. That the p21^{cp1} abundance was less responsive to protection by MG132 in HCMV-infected cells than in mock-infected cells is demonstrated by the significant difference ($P < 0.01$) in the slopes (determined by linear regression analysis) of the concentration effect of MG132 in HCMV-infected (0.800, $P < 0.01$) and mock-infected (1.964, $P < 0.01$) LU cells. These findings suggested that some additional MG132-insensitive pathway(s) may be involved in the proteolysis of p21^{cp1} in HCMV-infected cells. As the unidentified mechanism(s) seemed to be quantitatively more important in p21^{cp1} prote-

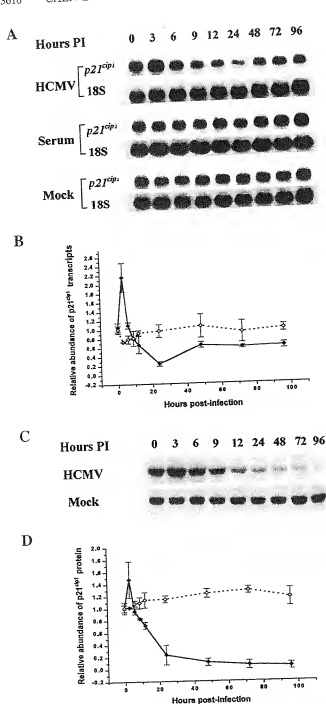
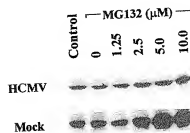


FIG. 1. (A) Northern blot analysis of the effect of HCMV infection (HCMV), serum growth factors (Serum), or mock infection (Mock) on p21^{cip1} RNA transcripts in density-arrested LU cells. The density-arrested cells were infected with HCMV at a multiplicity of 5 PFU/cell, arrested cells were infected with HCMV at a multiplicity of 5 PFU/cell, exposed to fresh FBS (10%), or mock infected. RNA was isolated at the times indicated, and 20 μ g of RNA from each lysate was applied to the gel. 18S rRNA (18S) was used as a loading standard. (B) Results of the representative experiment illustrated in panel A and of two additional independent experiments, evaluated by densitometric analysis and plotted as the abundance relative to the mock-infected control at 0 h p.i. with standard deviation shown (\diamond , p21^{cip1} RNA, HCMV-infected cells; \circ , p21^{cip1} RNA, mock-infected cells). (C) Western blot analysis of the abundance of p21^{cip1} after HCMV infection or mock infection. Parallel cultures of density-arrested LU cells were treated as

A. Western blot analysis



B. Densitometry

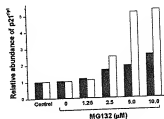


FIG. 2. Effect of MG132, an inhibitor of proteasome activity, on p21^{cip1} abundance in HCMV- or mock-infected, density-arrested LU cells. (A) Results of a representative experiment in which density-arrested LU cells were infected with HCMV (5 PFU/cell) or mock infected and then exposed to the indicated concentrations of MG132 beginning at 24 h p.i. Cell lysates were prepared at 30 h p.i. and 40 μ g of protein from each lysate was resolved by SDS-PAGE. The proteins were transferred to nitrocellulose and probed with antibodies against p21^{cip1}. Control, cells cultured in the absence of MG132 or its solvent, DMSO; 0, DMSO control, consisting of cells treated with the same concentration of DMSO (0.1%) present in the culture fluids containing the highest concentration of MG132 (10 μ M). (B) Change in abundance of p21^{cip1} after MG132 treatment, determined by densitometry of the results illustrated in panel A. The results for mock-infected cells (open bars) were calculated relative to the p21^{cip1} abundance in untreated mock-infected cells; the results for HCMV-infected cells (closed bars) were determined relative to the p21^{cip1} abundance in untreated HCMV-infected cells.

olysis in HCMV-infected cells than was proteasome-mediated degradation, we were interested in identifying the proteolytic mechanism(s) stimulated by HCMV infection.

The calpain inhibitors E64d and Z-Leu-Leu-H protect p21^{cip1} from degradation during HCMV infection. HCMV in-

in the legend to panel A. Whole-cell lysates were prepared at the indicated intervals, and 40 μ g of protein from each was resolved by SDS-PAGE. The proteins were transferred to nitrocellulose membrane and probed with antibodies against p21^{cip1}. The results of a representative experiment are illustrated. (D) The results of the experiment illustrated in panel C and two additional experiments, evaluated by densitometric analysis and plotted as the abundance relative to the mock-infected control at 0 h p.i. with standard deviation shown (\diamond , p21^{cip1} protein, HCMV-infected cells; \circ , p21^{cip1} protein, mock-infected cells).

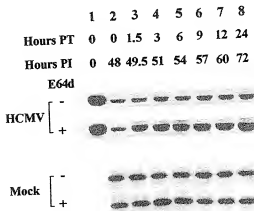
fection induces substantial increases in intracellular free Ca^{2+} (58) and in phospholipid degradation (1, 2, 81), raising the possibility that Ca^{2+} -activated neutral proteases (calpains) might be activated by HCMV infection and participate in the proteolysis of p21^{cp1}. Previous work has demonstrated that calpains are able to cleave cell cycle regulatory proteins, such as cyclin D1, cellular oncogene products (e.g., c-Mos, c-Jun, and c-Fos), and p53 (43, 59; for reviews, see references 21 and 28). The possibility that calpain-mediated proteolysis was involved in the degradation of p21^{cp1} in HCMV-infected cells was investigated first by examining the effect of the cell-permeable calpain inhibitors E64d (79) and Z-Leu-Leu-H (69) on p21^{cp1} levels following HCMV infection.

In initial experiments, we measured the effect of E64d (100 μM) on p21^{cp1} levels by Western blot analysis beginning at 48 h p.i., when p21^{cp1} was near its lowest level (Fig. 1) in HCMV-infected (5 PFU/cell), density-arrested LU cells. The abundance of p21^{cp1} was determined at selected intervals for 24 h following treatment with E64d. The results of a representative experiment are shown in Fig. 3. As noted previously, a substantial decrease in p21^{cp1} levels was observed at 48 h p.i. in HCMV-infected cells just prior to treatment with E64d (Fig. 3, compare lane 1 [lysate of mock-infected cells prepared at 0 h p.i.] to lane 2 [lysate of HCMV-infected cells in the absence of E64d prepared at 48 h p.i.]). Except at 3 h after treatment with E64d, when there was about a 1.5-fold increase in p21^{cp1} in the presence of E64d, the calpain inhibitor had little, if any, effect on the abundance of p21^{cp1} in mock-infected, density-arrested LU cells. In contrast, in HCMV-infected cells in the presence of E64d, a substantial accumulation of p21^{cp1} was observed, with a fourfold increase in the abundance of p21^{cp1} in HCMV-infected cells observed after 12 h of E64d treatment. In the absence of E64d, levels of p21^{cp1} in HCMV-infected cells remained at a constant low level. The maximum level of p21^{cp1} in the E64d-treated cells under these conditions was about 84% of that observed at 0 h. These data suggested that an E64d-sensitive proteolytic pathway was induced in HCMV-infected cells and was largely responsible for a substantial decrease in the abundance of p21^{cp1}.

The concentration effect of E64d on p21^{cp1} abundance was examined in HCMV (5 PFU/cell)- and mock-infected, density-arrested LU cells by addition of selected concentrations of E64d at 48 h p.i. Whole-cell extracts were prepared 6 h later and evaluated by Western blot analysis. The results of a representative experiment are shown in Fig. 4. Since E64d had little, if any, effect on p21^{cp1} levels in mock-infected cells even at a concentration of 100 μM , as demonstrated in Fig. 3, the data for mock-infected cells are not illustrated in Fig. 4. In HCMV-infected cells, protection of p21^{cp1} was observed at all concentrations evaluated (6.25 to 100 μM), while the quantitative effect was directly dependent on the concentration of E64d. Cells treated with the highest concentration of E64d (100 μM) demonstrated about 85% of the p21^{cp1} abundance measured in mock-infected cells over the same time period (Fig. 4B, compare the first and last columns).

Protection of p21^{cp1} by Z-Leu-Leu-H was evaluated by the same approach as described above. Z-Leu-Leu-H also protected p21^{cp1} in a concentration-dependent manner in HCMV (5 PFU/cell)-infected, density-arrested LU cells, with some protection provided by all concentrations of Z-Leu-Leu-H

A. Western blot analysis



B. Densitometry

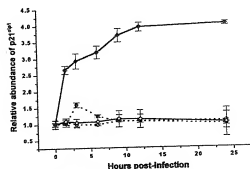
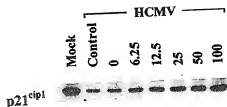


FIG. 3. Cumulative effect of the calpain inhibitor E64d (100 μM) on p21^{cp1} protein levels in HCMV- or mock-infected, density-arrested LU cells. (A) LU cells arrested by contact inhibition as described in Materials and Methods were infected with HCMV (5 PFU/cell) or mock infected and then exposed to E64d beginning at 48 h p.i. At the times indicated, whole-cell lysates were prepared, and 40 μg of protein from each lysate was analyzed by SDS-PAGE. The proteins were transferred to nitrocellulose and probed with antibodies against p21^{cp1}. The results of a representative experiment are shown. Hours PT (posttreatment) indicates the duration of E64d exposure. Hours PI indicates the time of harvest after infection. The exposure time for blots of lysates from HCMV-infected cells was about three times longer than that required for blots of lysates from mock-infected cells. Accordingly, to permit a quantitative comparison between the p21^{cp1} abundance at 0 h p.i. and at later times, lane 1 for the HCMV-infected cell lysates was loaded with a lysate from mock-infected cells not treated with E64d. (B) Accumulation of p21^{cp1} after E64d treatment, determined by densitometry of the data illustrated in panel A and from two additional experiments. The results for p21^{cp1} abundance in HCMV- and mock-infected cells with standard deviation shown are HCMV- and mock-infected cells, respectively (○, mock-infected cells; ●, mock-infected cells treated with E64d; ◇, HCMV-infected cells; ◆, HCMV-infected cells treated with E64d).

A. Western blot analysis



B. Densitometry

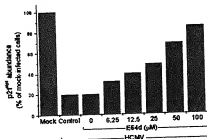
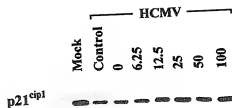


FIG. 4. Effect of E64d concentration on p21^{cip1} abundance in HCMV-infected cells. (A) LU cells were density arrested as described in Materials and Methods. HCMV infected (5 PFU/cell) or mock infected, and at 48 h p.i. treated with the indicated concentrations of E64d. Whole-cell lysates were prepared 6 h later, and 40 μg of protein from each lysate was resolved by SDS-PAGE. The proteins were transferred to nitrocellulose and probed with antibodies to p21^{cip1}. The results from a representative experiment are shown. Mock, mock-infected cells in the absence of drug; Control, untreated HCMV-infected cells; 0, DMSO control, consisting of HCMV-infected cells treated with the same concentration of DMSO (0.1%) contained in the highest concentration of E64d. (B) Densitometric analysis of the results shown in panel A.

evaluated. The results of a representative experiment are shown in Fig. 5. The levels of protection provided by Z-Leu-Leu-H were similar to those observed with E64d (Fig. 4). A concentration of 100 μM of Z-Leu-Leu-H resulted in detection of about 72% of the mock-infected levels of p21^{cip1} (Fig. 5B, compare the first and last columns), compared to 85% for cells treated with E64d. Z-Leu-Leu-H had little, if any, effect on the abundance of p21^{cip1} in mock-infected cells (data not shown).

Sensitivity of p21^{cip1} degradation to inhibition by E64d during the HCMV replication cycle. To determine the effect of E64d (100 μM) on p21^{cip1} levels throughout the time course of HCMV infection, density-arrested LU cell cultures were either infected with HCMV (5 PFU/cell) or mock infected and treated with E64d up to 12 h before harvest. For cells harvested at 12 h p.i. or before, the cells were treated with E64d from 1 h p.i. to the time of harvest (Fig. 6). E64d had little effect on p21^{cip1} levels in mock-infected cells, as previously noted (Fig. 3), and therefore the results for mock-infected cells are not shown. The results for HCMV-infected cells from a representative experiment are shown in Fig. 6A. In the absence

A. Western blot analysis



B. Densitometry

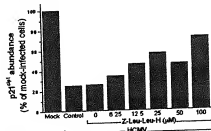
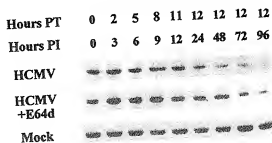


FIG. 5. Effect of Z-Leu-Leu-H concentration on p21^{cip1} abundance in HCMV-infected cells. (A) LU cells were density arrested as described in Materials and Methods, infected with HCMV (5 PFU/cell) or mock infected, and at 48 h p.i. treated with selected concentrations of Z-Leu-Leu-H. Whole-cell lysates were prepared 6 h later, and 40 μg of protein from each lysate was resolved by SDS-PAGE. The proteins were transferred to nitrocellulose and probed with antibodies to p21^{cip1}. The results illustrated are those from a representative experiment. Mock, Control, and 0 are defined in the legend to Fig. 4. (B) Densitometric analysis of the results illustrated in panel A.

of E64d, p21^{cip1} levels in HCMV-infected cells increased at 3 h and began to drop off by 6 h. By 72 and 96 h p.i., p21^{cip1} was only faintly detectable in the HCMV-infected cells in the absence of E64d. In the presence of E64d, p21^{cip1} levels in HCMV-infected cells were significantly ($P < 0.05$) greater (from 3 h to 96 h p.i., except at 12 and 96 h, when the difference, although greater, was not significant) than the levels observed in the absence of the calpain inhibitor (Fig. 6B). As a result of E64d treatment, p21^{cip1} levels remained at or above the preinfection levels through 9 h p.i. and within 85% of the preinfection levels through 24 h. After 48 h, p21^{cip1} levels in E64d-treated, HCMV-infected cells remained well above the levels in the absence of the calpain inhibitor, but the p21^{cip1} abundance dropped progressively below the preinfection levels. Considered together, these data suggested that calpain-mediated proteolysis contributed substantially to the degradation of p21^{cip1} in HCMV-infected cells from 3 h through the late phase of HCMV infection.

Calpain is activated by HCMV infection. The level of calpain activity is normally very low in unstimulated cells due to the presence of the endogenous calpain inhibitor calpastatin (21). The findings that treatment of HCMV-infected cells with E64d or Z-Leu-Leu-H led to stabilization and accumulation of p21^{cip1} suggested that calpain is activated during HCMV in-

A. Western blot analysis



B. Densitometry

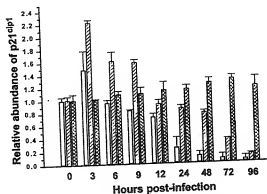


FIG. 6. Time course for the effect of E64d on p21^{cp1} abundance in HCMV-infected, density-arrested cells. (A) LU cells density arrested as described in Materials and Methods were infected with HCMV (5 PFU/cell) or mock infected; at the intervals indicated, beginning 1 h p.i., subsets of the cells were treated with E64d (100 μ M). Whole-cell lysates were prepared at the times indicated (up to 12 h posttreatment [PT]), and 40 μ g of protein from each lysate was resolved by SDS-PAGE. The proteins were transferred to nitrocellulose and probed with antibodies to p21^{cp1}. The results of a representative experiment are illustrated. (B) Densitometric analysis of the results shown in panel A and two additional experiments with standard deviation. Open bars, HCMV-infected cells; diagonally striped bars, HCMV-infected cells treated with E64d; cross-hatched bars, mock-infected cells.

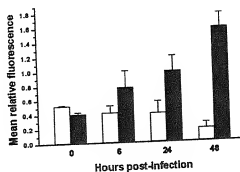
fection. To investigate this possibility, we used the fluorogenic calpain substrate Boc-Leu-Met-CMAC to measure calpain activity in density-arrested LU cells (67). Density-arrested LU cells were infected with HCMV (5 PFU/cell) or mock infected and exposed to Boc-Leu-Met-CMAC (10 μ M) 15 min before assay. The cells were harvested (see Materials and Methods), and the relative fluorescence was determined. The results (Fig. 7A) demonstrated that fluorescence from the product (AMC) of cleavage of the fluorogenic substrate fell gradually in mock-infected cells during the 48-h course of these experiments. HCMV infection, however, induced a substantial increase in calpain activity. An increase in AMC fluorescence was observed by 6 h p.i. in HCMV-infected cells. After 24 h, the AMC fluorescence in HCMV-infected cells increased to a value

about 2.5-fold greater than that observed in mock-infected cells. The relative fluorescence observed in the HCMV-infected cells increased further by 48 h p.i., when it was about eightfold greater than the fluorescence in mock-infected cells. The increases in calpain activity from 24 and 48 h were significant ($P < 0.05$). These data closely reflect the time course for the changes in the abundance of p21^{cp1} following HCMV infection in our earlier work (19) and in this study. In these studies, a decrease in p21^{cp1} levels was first detected at 6 h p.i., and the abundance of p21^{cp1} continued to decrease through 48 h p.i.

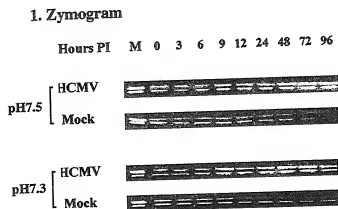
Zymography. Since casein is a well-established target of calpain-mediated proteolysis (87), we used casein zymography to verify that calpain was activated in HCMV-infected cells, and to determine which calpain isoenzymes were activated, and to extend the time course data for calpain activity. Density-arrested LU cells were infected with HCMV (5 PFU/cell) or mock infected, harvested at times from 0 to 96 h p.i., and evaluated by casein zymography. The results of casein zymography to evaluate cellular proteolysis are illustrated in Fig. 7B. When both of the ubiquitous calpains are activated, μ -calpain is observed as the upper band, while m-calpain is observed at a slightly lower position in the nondenaturing gels (89). The intensity of the bands was related linearly to the amount of protease activity. Since μ - and m-calpain have slightly different pH optima, duplicate gels were incubated at pH 7.5 or 7.3 to evaluate quantitative changes in isoenzyme activity. There was substantial activity of both ubiquitous calpains at each pH shown when both calpains were activated. The results for a representative experiment from this series of experiments (Fig. 7B) demonstrated that calpain activity gradually decreased in mock-infected cells during the 96-h time course of the experiment. In contrast, HCMV infection induced an increase in both μ - and m-calpain activities by 6 to 9 h p.i. (Fig. 7B, part 2). By 24 h, the calpain activity of HCMV-infected cells was about fourfold (μ -calpain) or fivefold (m-calpain) greater than that of mock-infected cells. The enzyme activity continued to increase and reached its maximum level at 72 h p.i. when it was about 23-fold (μ -calpain) or 25-fold (m-calpain) greater than that observed in mock-infected cells. The increases in calpain activities from 12 to 96 h were significant ($P < 0.05$) relative to the preinfection levels. Overall, the changes in calpain activity measured by casein zymography were remarkably similar to those obtained using the fluorogenic calpain substrate Boc-Leu-Met-CMAC in intact cells (Fig. 7A) and indicate that HCMV infection was activating both μ - and m-calpain.

To determine if the increased calpain activity observed in Fig. 7A and B was a result of increased expression of calpains or a decrease in the abundance of calpastatin, the abundance of these proteins was measured by Western blot analysis (Fig. 7C) in cells treated in parallel with those used to measure calpain activity (Fig. 7B). HCMV infection had little, if any, detectable effect on the abundance of μ -calpain or calpastatin through 96 h p.i. m-Calpain levels demonstrated a very slight increase from 6 h to 72 h p.i. over the abundance observed in mock-infected cells, and by 96 h, HCMV- and mock-infected cells demonstrated similar levels of m-calpain. Given the very small difference in the abundance of m-calpain in HCMV- and mock-infected cells, it was questionable if this difference had any significance. Mock infection had little, if any, effect on the

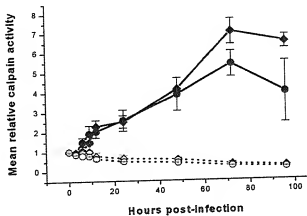
A. Calpain activity-Fluorescence



B. Calpain activity-Zymogram

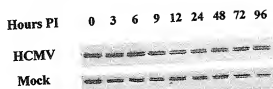


2. Densitometry

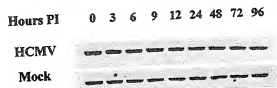


C. Western blot analysis

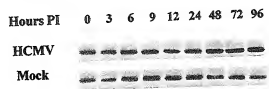
1. m-Calpain



2. μ -Calpain

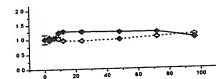


3. Calpastatin

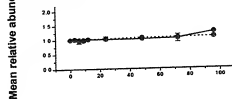


4. Densitometry

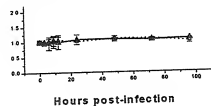
a. m-Calpain



b. μ -Calpain



c. Calpastatin



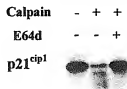
cellular levels of μ -calpain, m-calpain, or calpastatin during the time course of this series of experiments. Thus, it is unlikely that the substantial increase in calpain activity observed in HCMV-infected cells was a result of a decrease in calpastatin abundance or of increases in the abundance of the ubiquitous calpains.

Calpain cleaves p21^{cp1}. Although a number of proteins are now recognized as targets of calpain-mediated proteolysis (21), p21^{cp1} has previously not been included among them. Accordingly, we were interested in directly testing the sensitivity of p21^{cp1} to calpain-mediated proteolysis by incubating purified recombinant p21^{cp1}, obtained from J. Wade Harper (41) or Hengming Ke (53), with either purified μ -calpain or m-calpain and evaluating the products by Western blot analysis. We also prepared recombinant p21^{cp1} by using the expression plasmid pET-p21, provided by J. Wade Harper (32, 41). Incubation of any of the purified p21^{cp1} preparations with purified μ -calpain or m-calpain resulted in the rapid cleavage of p21^{cp1}, as illustrated with findings for μ -calpain in Fig. 8A. Two prominent p21^{cp1} fragments were observed after incubation with calpain, consistent with the results from earlier studies indicating that calpain typically cleaves its target proteins at a limited number of sites (for a review, see reference 78). Inclusion of E64d in the digestion inhibited the proteolysis of p21^{cp1} (Fig. 8A). To investigate if either of the ubiquitous calpains was removing or modifying the availability of the epitope that was the target of the anti-p21^{cp1} antibody used in the Western blot analysis, we incubated p21^{cp1} and casein separately with either μ -calpain or m-calpain and examined the proteolytic products by SDS-PAGE. The location of polypeptides in the gels was demonstrated by Coomassie brilliant blue staining. To minimize the loss of small peptides, electrophoresis of proteins applied to these gels was for a period of time shorter than used for Western blot analysis (Fig. 8A). A limited number of cleavage products was observed in the Coomassie blue-stained gels (Fig. 8B) for either casein or p21^{cp1} incubated with either of the ubiquitous calpains, as had been observed by Western blot analysis (Fig. 8A). Thus, p21^{cp1} appeared to be a target for proteolysis mediated by either μ - or m-calpain.

DISCUSSION

Involvement of p21^{cp1} in cell cycle progression in HCMV-infected cells. Progression through the cell cycle is regulated by

A. Western blot



B. Coomassie brilliant blue stain

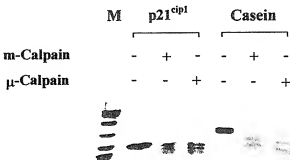


FIG. 8. Cleavage of purified recombinant p21^{cp1} by purified calpain. (A) p21^{cp1} (0.4 μ g) was incubated with μ -calpain (0.004 U) for 30 min at 30°C in the presence or absence of E64d (100 μ M), and the resulting products were analyzed by Western blot analysis as described in Materials and Methods. The data illustrated are from a representative experiment. (B) p21^{cp1} (4 μ g) or casein (3 μ g) was incubated with 0.04 U of μ -calpain or m-calpain for 30 min at 30°C, and the products of the digestion were examined by SDS-PAGE and Coomassie brilliant blue staining. M, Rainbow colored protein molecular weight markers of 97.4, 66, 46, 30, 21.5, and 14.3 kDa (Amersham Life Science). The data illustrated are from a representative experiment.

mechanisms that are beginning to be understood (31, 73, 74). Induction of cyclin E-associated kinase activity is essential for progression through the G₁ phase of the cell cycle (for a review, see reference 49) and preparation of the cell for DNA

FIG. 7. (A) The time course for calpain activity in HCMV (5 PFU/cell)-infected (solid bars) and mock-infected (open bars) density-arrested cells. Calpain activity was measured by exposing the cells to the cell-permeant fluorogenic calpain substrate Boc-Leu-Met-CMAC (18 μ M) for 15 min before the fluorescence intensity was measured for equal numbers of HCMV- and mock-infected cells, using an SLM 4800S spectrofluorometer. Excitation was at 380 nm; emission was at 460 nm. The polarizers were set at 0° and 50°. The data plotted are the means (\pm standard deviation) of the findings from three independent experiments. (B) 1, representative casein zymogram. Density-arrested LU cells were either infected with HCMV (5 PFU/cell) or mock infected. The cells were harvested at the times indicated and processed for zymography as described in Materials and Methods. Since calpain activity is pH dependent, the gels were incubated in incubation buffer at either pH 7.5 (optimal for μ -calpain) or pH 7.3 (optimal for m-calpain). Purified μ - and m-calpain (0.1 U of each) were added to the sample in lane 1 as a positive control. The upper band in each lane corresponds to μ -calpain, whereas the lower band corresponds to m-calpain (89). 2, data for calpain activity based on the means (\pm standard deviation) of the intensity of bands at each time relative to the intensity of the corresponding band at 0 h p.i. for the zymograms shown above (\blacklozenge , μ -calpain, HCMV-infected cells; \circ , μ -calpain, mock-infected cells; \bullet , m-calpain, HCMV-infected cells; \circ , m-calpain, mock-infected cells). (C) 1 to 3, abundances of m-calpain, μ -calpain, and calpastatin in parallel cell cultures as determined by Western blot analysis (results of a representative experiment); 4, densitometric analysis of the results shown in panel A and two additional experiments with standard deviation. Solid symbols (\blacklozenge , m-calpain; \bullet , μ -calpain; \blacktriangle , calpastatin) and line represent the means of the results for HCMV-infected cells; open symbols (\circ , m-calpain; \circ , μ -calpain; Δ , calpastatin) and dashed line represent the results for mock-infected cells. Error bars not shown are smaller than the symbol.

synthesis. Data from this laboratory (19) and others (45) have demonstrated that HCMV infection substantially increases E kinase activity in arrested cells. In density-arrested cells, E kinase activity is first substantially increased at 24 h p.i., reaches its maximum level by 72 h, and is still detectable at 96 h (19), well into the late phase of HCMV replication.

Several mechanisms have been identified that regulate the activity of E kinase. For example, an increased cellular abundance of cyclin E is associated with activation of E kinase (reference 65 and references therein). HCMV infection has been shown to transcriptionally activate the expression of cyclin E, but the time course for the increase in E kinase activity is delayed relative to the changes in the cellular abundance of cyclin E (19). The cellular levels of cyclin E protein in density-arrested cells increase substantially by 12 h p.i., peak by 24 h, and are in decline by 48 h (19). Accordingly, not only does activation of E kinase lag behind the increase in cyclin E abundance, but E kinase remains activated longer than would be anticipated based solely on cyclin E abundance (19). For example, despite the decrease in cyclin E levels at 48 h and continuing reduction thereafter, E kinase activity increases remarkably at 48 h p.i., peaks at 72 h, and still remains high at 96 h p.i. The lack of congruity in the data for cyclin E abundance and E kinase activity suggest that other mechanisms are involved in the regulation of E kinase activity in HCMV-infected cells.

The activity of cyclin E-Cdk2 complexes is also regulated by CKIs, including p21^{cp1}. In fact, it has been suggested that p21^{cp1} establishes a threshold in the nucleus (34, 44, 75, 86), which must be exceeded if E kinase activity is to develop from cyclin E-Cdk2 complexes. Increases in E kinase activity in HCMV-infected, density-arrested cells closely parallel a substantial reduction in the abundance of p21^{cp1} by 24 h p.i. (19). Our earlier results suggest that the delay in activation of E kinase activity after the increase in cyclin E abundance and the persistence of the cyclin E-associated kinase activity after cyclin E levels are sharply reduced are related to the effects of the CKIs p21^{cp1} and p27^{kip1} (19). Both p21^{cp1} and p27^{kip1} are abundant in quiescent LU cells, contributing to the observed delay between increasing cyclin E levels and activation of E kinase until cyclin E-Cdk2 complexes exceed the threshold established by p21^{cp1} and p27^{kip1}. Later, as cyclin E levels are falling, E kinase remains active because HCMV infection stimulates reduction of both p21^{cp1} and p27^{kip1}, particularly of p21^{cp1} (19). Thus, the initial phase of activation of E kinase activity is associated with a substantial increase in cyclin E levels and decrease in p21^{cp1} levels, while a substantial and sustained decrease in p21^{cp1} is associated with the persistence of E kinase activity observed during HCMV infection (Fig. 1D).

Proteolysis of p21^{cp1} in mock- and HCMV-infected cells. The abundance of each protein in a living cell depends on the balance between its synthesis and degradation, and therefore intracellular proteolysis is of critical importance in maintaining functional concentrations. In fact, many regulatory proteins are subject to proteolytic degradation, which allows cells to rapidly adjust their concentration. Reduction of p21^{cp1} levels in noninfected cells is reported to be mediated by ubiquitination and proteasome degradation (10, 35, 38, 85). Indeed, in mock-infected LU cells, proteasome degradation was the predominant pathway for p21^{cp1} proteolysis (Fig. 2). In contrast,

the present results indicate that in HCMV-infected cells, the proteasome is responsible for little, whereas calpain activity is responsible for much, of the degradation of p21^{cp1} observed during the progression of the virus infection. The early decrease in p21^{cp1} abundance appears to be related in part to decreasing RNA levels (Fig. 1) and in part to increasing calpain-mediated degradation (Fig. 6). Since the half-life of p21^{cp1} is reported to be approximately 2 h (46), a decrease in RNA levels would predictably result in lower levels of the protein. When the activity of calpain was inhibited at these early times (e.g., 9 h p.i.), p21^{cp1} levels rose to 158% of the preinfection levels, compared with 83.1% in the absence of calpain inhibitors (Fig. 6). At 24 h p.i., when a substantial increase in E kinase activity is first detected, p21^{cp1} levels are at 23% of their preinfection levels, but when calpain activity is blocked, p21^{cp1} levels are at 85% of their preinfection levels. This level of p21^{cp1} is similar to the levels observed in absence of calpain inhibition at 9 and 12 h p.i. (83 and 72%, respectively), when E kinase activity is at a very low level (19). Thus, calpain-mediated mechanisms appear to be responsible for the degradation of a substantial portion of p21^{cp1}, even at early times in HCMV replication. At later times (after 48 h), when RNA levels are relatively constant, calpain-mediated mechanisms are largely responsible for the continuing decrease in p21^{cp1} abundance. During this period (48 and 72 h p.i.), inhibition of calpain-catalyzed proteolysis produced cellular levels of p21^{cp1} that were from 77.5 to 36.4% of the preinfection levels, whereas in the absence of calpain inhibitors, the level of p21^{cp1} fell to 10.8 and 6.2%, respectively, of the preinfection level (Fig. 6). Thus, HCMV-induced activation of calpain is important at both early and late times in controlling the abundance of p21^{cp1} and, accordingly, the activity of the cyclin E-associated kinase. The calpain effect seems particularly noteworthy in extending the activation of E kinase well into the late phase of HCMV replication. Since E kinase activity has been shown to be necessary for high infectious yields of HCMV (18), it is tempting to speculate that the sustained calpain activity and persistent low levels of p21^{cp1} support durable activation of E kinase and production of high yields of infectious HCMV. Indeed, preliminary experiments indicate that a single exposure to Z-Leu-Leu-H (100 μ M) is sufficient to reduce HCMV infectious yields at 96 h PI by 99.7% ($P < 0.05$).

Several experimental approaches support the view that calpain activity and p21^{cp1} levels are closely coupled in HCMV-infected cells. Calpain activity increases in a temporal pattern that is consistent with the time courses for the decrease in p21^{cp1} abundance and the increase in E kinase activity in HCMV-infected cells (compare Fig. 7 with Fig. 1; see also reference 19). The time course for E64d protection of p21^{cp1} (Fig. 3) closely parallels the decrease in p21^{cp1} (Fig. 1) and the increase in calpain activity (Fig. 7). The presence of inhibitors of calpain activity (E64d, Z-Leu-Leu-H) protects up to about 80% of p21^{cp1} (Fig. 4 and 5). The concentration effect of E64d in providing protection of p21^{cp1} in HCMV-infected LU cells is in the range (6.25 to 100 μ M) observed in earlier studies to protect other targets of calpain-mediated proteolysis (54). In cell-free studies, purified calpain degrades purified recombinant p21^{cp1} (Fig. 8). Considered together, these results indicate that calpain activity is largely responsible for the substantial decrease in p21^{cp1} observed during HCMV infection.

ecules in the limited cell cycle progression induced by productive HCMV infection.

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REFERENCES

- AbuBakar, S., I. Boldogh, and T. Albrecht. 1990. Stimulation of [³H] release from [³H]-arachidonic acid prelabeled cells. *Arch. Virol.* 113:255-266.
- AbuBakar, S., I. Boldogh, and T. Albrecht. 1990. Human cytomegalovirus stimulates arachidonic acid metabolism through pathways that are affected by inhibitors of phospholipase A2 and protein kinase C. *Biochem. Biophys. Res. Commun.* 166:952-959.
- Albrecht, T., and T. H. Weller. 1980. Heterogeneous morphologic features of plaques induced by five strains of human cytomegalovirus. *Am. J. Clin. Pathol.* 73:648-654.
- Albrecht, T., I. Boldogh, and M. P. Fons. 1992. Receptor-initiated activation of cells and their oncogenes by herpes-family viruses. *J. Invest. Dermatol.* 98(Suppl.):295-355.
- Albrecht, T., I. Boldogh, M. Fons, C. H. Lee, S. AbuBakar, J. M. Russell, and W. W. Au. 1989. Cell-activation responses to cytomegalovirus infection: relationship to the phasing of CMV replication and to the induction of cellular damage. *Subcell. Biochem.* 15:157-202.
- Albrecht, T., T. Cavallo, N. L. Cole, and K. Graves. 1980. Cytomegalovirus: development and progression of cytopathic effects in human cell culture. *Lab. Invest.* 42:1-7.
- Albrecht, T., M. Nachtigal, S. C. St. Jeor, and F. Rapp. 1976. Induction of cellular DNA synthesis and increased mitotic activity in Syrian hamster embryo cells acutely infected with human cytomegalovirus. *J. Gen. Virol.* 39:167-177.
- Afford, C. A., S. Stagno, R. F. Pass, and W. J. Britt. 1990. Congenital and perinatal cytomegalovirus infections. *Rev. Infect. Dis.* 12(Suppl.):S745-S753.
- Arora, A. S., P. de Groen, Y. Emori, and G. J. Gores. 1996. A cascade of degradative hydrolytic activity contributes to hepatocyte necrosis during anoxia. *Am. J. Physiol.* 272:G238-G245.
- Blagosklonny, M. V., G. S. Wu, S. Omura, and W. S. el-Deiry. 1996. Proteasome-dependent regulation of p21^{WAF1/CIP1} expression. *Biochem. Biophys. Res. Commun.* 227:564-569.
- Boldogh, I., S. AbuBakar, and T. Albrecht. 1990. Activation of proto-oncogenes: an immediate early event in human cytomegalovirus infection. *Science* 247:561-564.
- Boldogh, I., S. AbuBakar, C. Z. Deng, and T. Albrecht. 1991. Transcriptional activation of cellular oncogenes *fos*, *jun*, and *myc* by human cytomegalovirus. *J. Virol.* 65:1568-1571.
- Boldogh, I., S. AbuBakar, D. Millonig, C. Z. Deng, and T. Albrecht. 1991. Cellular oncogene activation by human cytomegalovirus: lack of correlation with virus infectivity and IE gene expression. *Arch. Virol.* 118:163-177.
- Boldogh, I., M. P. Fons, and T. Albrecht. 1993. Increased activity of DNA-binding proteins in nuclei of human cytomegalovirus-infected MRC-5 cells. *Biochem. Biophys. Res. Commun.* 197:1505-1510.
- Bonin, L. R., and J. K. McLaughlin. 1997. Human cytomegalovirus IE2 86-kilodalton protein binds p53 but does not abrogate G₁ checkpoint function. *J. Virol.* 71:5861-5870.
- Bowman, L. H., B. Rabin, and D. Schlessinger. 1981. Multiple ribosomal RNA cleavage pathways in mammalian cells. *Nucleic Acids Res.* 9:4951-4966.
- Bresnahan, W. A., T. Albrecht, and E. A. Thompson. 1998. The cyclin E promoter is activated by human cytomegalovirus 86-kDa immediate early protein. *J. Biol. Chem.* 273:22075-22082.
- Bresnahan, W. A., I. Boldogh, P. Chi, E. A. Thompson, and T. Albrecht. 1997. Inhibition of cellular Cdk2 activity blocks human cytomegalovirus replication. *Virology* 231:239-247.
- Bresnahan, W. A., I. Boldogh, E. A. Thompson, and T. Albrecht. 1996. Human cytomegalovirus inhibits cellular DNA synthesis and arrests productively infected cells in late G₁. *Virology* 224:150-160.
- Bresnahan, W. A., E. A. Thompson, and T. Albrecht. 1997. Human cytomegalovirus infection results in altered Cdk2 subcellular localization. *J. Gen. Virol.* 78:1993-1997.
- Carroll, E., and M. Molinari. 1998. Calpain: a protease in search of a function? *Biochem. Biophys. Res. Commun.* 247:193-203.
- Carillo, S., M. Parlati, A. M. Stef, L. Jaric-Encineta, F. Poulat, P. Beria, and M. Piechaczyk. 1996. PEST motifs are not required for rapid calpain-mediated proteolysis of c-fos protein. *Biochem. J.* 313:245-251.
- Castillo, J. P., A. D. Yurochko, and T. F. Kowalik. 2000. Role of human cytomegalovirus immediate-early proteins in cell growth control. *J. Virol.* 74:6828-6837.
- Chen, I.-T., M. L. Smith, P. M. O'Connor, and A. J. Fornace, Jr. 1995. Direct interaction of Gadd45 with PCNA and evidence for competitive interaction of Gadd45 and p21^{WAF1/CIP1} with PCNA. *Oncogene* 11:1931-1937.
- Chomczynski, P. 1993. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *BioTechniques* 15:532-534.
- Ciechanover, A. 1994. The ubiquitin-proteasome proteolytic pathway. *Cell* 79:13-21.
- Coocian, S. A., and D. R. Hathaway. 1984. Effect of α -phosphatidylinositol on a vascular smooth muscle Ca²⁺-dependent protease. Reduction of the Ca²⁺ requirement for autolysis. *J. Biol. Chem.* 259:11627-11630.
- Crowl, B. E., and G. N. DelMarino. 1991. Calcium-activated neutral protease (calpain) system: structure, function, and regulation. *Physiol. Rev.* 71:813-847.
- de Jong, M. D., G. J. Galasso, B. Gazzard, P. D. Griffiths, D. A. Jabs, E. R. Kern, and S. A. Spector. 1998. Summary of the 2nd International Symposium on Cytomegalovirus. *Antiviral Res.* 39:141-160.
- Dittmer, D. A., and E. S. Mocarski. 1997. Human cytomegalovirus infection inhibits G₁/S transition. *J. Virol.* 71:1629-1634.
- Draetta, G. F. 1994. Mammalian G1 cyclins. *Curr. Opin. Cell Biol.* 6:842-846.
- Dynlacht, B. D., C. Ngwu, J. Winston, E. C. Swindle, S. J. Elledge, E. Harlow, and J. W. Harper. 1997. Purification and analysis of CIP/KIP proteins. *Meth. Enzymol.* 283:200-244.
- El-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kluetz, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell* 75:817-825.
- Elledge, S. J., and J. W. Harper. 1994. Cdk inhibitors: on the threshold of checkpoints and development. *Curr. Opin. Cell Biol.* 6:487-492.
- Elledge, S. J., and J. W. Harper. 1998. The role of protein stability in the cell cycle and cancer. *Biochem. Biophys. Acta* 1377:M61-M70.
- Flores-Rozas, H., Z. Kelman, F. B. Dean, Z. Q. Pan, J. W. Harper, S. J. Elledge, M. O'Donnell, and J. Hurwitz. 1994. Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase δ holoenzyme. *Proc. Natl. Acad. Sci. USA* 91:8655-8659.
- Fortunato, E. A., M. H. Sommer, K. Yoder, and D. H. Spector. 1997. Identification of domains within the human cytomegalovirus major immediate-early 86-kilodalton protein and the retinoblastoma protein required for physical and functional interaction with each other. *J. Virol.* 71:8176-8185.
- Fukuchi, K., S. Tomoyasu, T. Nakamaki, N. Tsuruta, and K. Gomi. 1998. DNA damage induces p21 protein expression by inhibiting ubiquitination in ML-1 cells. *Biochem. Biophys. Acta* 1404:405-411.
- Gervais, J. L., P. Seth, and H. Zhang. 1998. Cleavage of CDK inhibitor p21^{CIP1/WAF1} by caspases is an early event during DNA damage-induced apoptosis. *J. Biol. Chem.* 273:19207-19212.
- Hagemeier, C., R. Caswell, G. Hayhurst, J. Sinclair, and T. Kouzarides. 1994. Functional interaction between the HCMV IE2 transactivator and the retinoblastoma protein. *EMBO J.* 13:2897-2903.
- Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Elledge. 1993. The p21 Cdk-inhibiting protein CIP1 is a potential inhibitor of G1 cyclin-dependent kinases. *Cell* 75:805-816.
- Harper, J. W., S. J. Elledge, K. Keyomarsi, B. Dynlacht, L. H. Tsai, P. Zhang, S. Dobrowski, C. Bai, L. Connell-Crowley, E. Swindle, M. P. Fox, and N. Wei. 1995. Inhibition of cyclin-dependent kinases by p21. *Mol. Biol. Cell* 6:357-400.
- Hirai, S., H. Kawasaki, M. Yaniv, and K. Suzuki. 1991. Degradation of transcription factors, c-Jun and c-Fos, by calpain. *FEBS Lett.* 287:57-61.
- Hunter, T., and J. Pines. 1994. Cyclins and cancer II: cyclin D and CDK inhibitors come of age. *Cell* 79:573-582.
- Jaul, F. M., J. M. Jaul, F. Ruch, E. A. Fortunato, C. Clark, J. Corbelli, D. D. Richmond, and D. H. Spector. 1995. Cytomegalovirus infection induces high levels of cyclins phosphorylated Rb and p53, leading to cell cycle arrest. *J. Virol.* 69:6697-6704.
- Johannesen, L. E., S. L. Knaflitz, and I. H. Madhus. 1999. Epidermal growth factor increases the level of cyclin-dependent kinase (CDK) inhibitor p21/CIP1 (CDK-interacting protein 1) in A431 cells by increasing the half-lives of the p21/CIP1 transcript and the p21/CIP1 protein. *Biochem. J.* 337:599-606.
- Johnson, R. A., A. D. Yurochko, E. E. Ponn, L. Zhu, and E. S. Huang. 1999. Domain mapping of the human cytomegalovirus IE1-72 and cellular p107

- protein-protein interaction and the possible functional consequences. *J. Gen. Virol.* 80:1293-1303.
48. Kearsey, J. M., P. J. Coates, A. R. Prescott, E. Warbrick, and P. A. Hall. 1995. Gadd45 is a nuclear cell cycle regulated protein which interacts with p21^{ras}. *Oncogene* 11:1673-1683.
 49. Keyomarsi, K., and T. W. Herlikey. 1997. The role of cyclin E in cell proliferation, development and cancer. *Prog. Cell Cycle Res.* 3:171-191.
 50. Kihlström, M. H., and K. H. Vousden. 1997. Proteolytic cleavage of human p53 by calpain: a potential regulator of protein stability. *Mol. Cell. Biol.* 17:460-468.
 51. Li, R., S. Waga, G. J. Hannon, D. Beach, and B. Stillman. 1994. Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. *Nature* 371:534-537.
 52. Lu, M., and T. Shenk. 1996. Human cytomegalovirus infection inhibits cell cycle progression at multiple points, including the transition from G₁ to S. *J. Virol.* 70:8550-8557.
 53. Mayruse, D. R., M. A. Nichols, Y. Xiong, and H. Ke. 1996. Purification and crystallization of cyclin-dependent kinase inhibitor p21. *Protein Sci.* 5:1928-1930.
 54. Mellgren, R. L. 1997. Evidence for participation of a calpain-like cysteine protease in cell cycle progression through late G₁ phase. *Biochem. Biophys. Res. Commun.* 236:555-558.
 55. Murphy, E. A., D. N. Strehlow, J. A. Nelson, and M. Stinski. 2000. The human cytomegalovirus IE85 protein can block cell cycle progression after inducing transition into the S phase of permissive cells. *J. Virol.* 74:7198-7218.
 56. Murray, E. J., G. V. Bentley, M. S. Grisanti, and S. S. Murray. 1998. The ubiquitin-proteasome system and cellular proliferation and regulation in osteoblastic cells. *Exp. Cell Res.* 242:460-469.
 57. Noda, A., Y. Ning, S. F. Venable, O. M. Pereira-Smith, and J. R. Smith. 1994. Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp. Cell Res.* 211:690-98.
 58. Nukta, M., D. Eaton, O. S. Steinhardt, and T. Albrecht. 1987. Ca²⁺ responses in cytomegalovirus-infected fibroblasts of human origin. *Virology* 157:259-267.
 59. Pahl, H. L., and P. A. Baeuerle. 1996. Control of gene expression by proteolysis. *Curr. Opin. Cell Biol.* 8:340-347.
 60. Pan, Z. Q., J. T. Reardon, L. Li, B. Flores-Rozas, R. Legerski, A. Sancar, and J. Hurwitz. 1995. Inhibition of nucleotide excision repair by the cyclin-dependent kinase inhibitor p21. *J. Biol. Chem.* 270:22008-22016.
 61. Perez, J. L. 1997. Resistance to antivirals in human cytomegalovirus: mechanisms and clinical significance. *Microbiologia* 13:343-352.
 62. Podust, V. N., L. M. Podust, F. Goubin, B. Ducommun, and U. Huhscher. 1995. Mechanism of inhibition of proliferating cell nuclear antigen-dependent DNA synthesis by the cyclin-dependent kinase inhibitor p21. *Biochemistry* 34:8869-8875.
 63. Poma, E. E., T. F. Kowalik, L. Zhu, J. H. Sinclair, and E. S. Huang. 1996. The human cytomegalovirus IE1-72 protein interacts with the cellular p107 protein and relieves p107-mediated transcriptional repression of an E2F-responsive promoter. *J. Virol.* 70:7867-7877.
 64. Rechsteiner, M., and S. W. Rogers. 1996. PEST sequences and regulation by proteolysis. *Trends Biochem. Sci.* 21:267-271.
 65. Resnick, D., M. Gossen, H. Bujard, and S. I. Reed. 1994. Acceleration of the G₁/S phase transition by expression of cyclins D1 and E with an inducible system. *Mol. Cell. Biol.* 14:1669-1679.
 66. Rogers, S., R. Wells, and M. Rechsteiner. 1986. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* 234:364-368.
 67. Rosser, B. G., S. P. Powers, and G. J. Geres. 1993. Calpain activity increases in hepatocytes following addition of ATP. Demonstration of a novel fluorescent assay. *J. Biol. Chem.* 268:23593-23600.
 68. Rubin, R. H. 1990. Impact of cytomegalovirus infection on organ transplant recipients. *Rev. Infect. Dis.* 12(Suppl.):S754-S766.
 69. Saito, Y., S. Tsubuki, H. Ito, and S. Kawashima. 1990. The structure-function relationship between peptide aldehyde derivatives on initiation of neurite outgrowth in PC12 cells. *Neurosci. Lett.* 120:1-4.
 70. Santella, L. 1998. The role of calcium in the cell cycle: facts and hypotheses. *Biochem. Biophys. Res. Commun.* 244:317-324.
 71. Santella, L., K. Kyoizumi, L. De Riso, and E. Carafoli. 1998. Calcium, protease action, and the regulation of the cell cycle. *Cell Calcium* 23:123-130.
 72. Schooley, R. T. 1990. Cytomegalovirus in the setting of infection with human immunodeficiency virus. *Rev. Infect. Dis.* 12(Suppl.):S811-S819.
 73. Sherr, C. J. 1993. Mammalian G₁ cyclins. *Cell* 73:1059-1065.
 74. Sherr, C. J. 1994. G₁ phase progression: cycling on cue. *Cell* 79:551-555.
 75. Sherr, C. J., and J. M. Roberts. 1995. Inhibitors of mammalian G₁ cyclin-dependent kinases. *Genes Dev.* 9:1149-1163.
 76. Sinclair, J., J. Baillie, L. Bryant, and R. Caswell. 2000. Human cytomegalovirus mediates cell cycle progression through G₁ to enter S phase in terminally differentiated cells. *J. Gen. Virol.* 81:1553-1565.
 77. Spier, E., E. S. Huang, R. Modali, M. B. Leon, F. Shaw, T. Finke, and S. E. Epstein. 1995. Interaction of human cytomegalovirus with p53: possible role in coronary restenosis. *Scand. J. Infect. Dis.* 27(Suppl. 99):78-81.
 78. Suzuk, K., and H. Sorimachi. 1998. A novel aspect of calpain activation. *FEBS Lett.* 433:1-4.
 79. Tami, M., K. Matsumoto, S. Omura, L. Koyama, Y. Ozawa, and K. Hanada. 1986. *In vitro* and *in vivo* inhibition of cysteine proteinases by EST, a new analog of E-64. *J. Pharmacobiodyn.* 9:672-677.
 80. Tsai, H. L., G. H. Kou, S. C. Chen, C. W. Wu, and Y. S. Lin. 1996. Human cytomegalovirus immediate-early protein IE2 tethers a transcriptional repression domain to p53. *J. Biol. Chem.* 271:3534-3540.
 81. Vally-Nagy, T., Z. Bandi, I. Boldogh, and T. Albrecht. 1988. Hydrolysis of inositol lipid: an early signal of human cytomegalovirus infection. *Arch. Virol.* 101:199-207.
 82. Waga, S., G. J. Hannon, D. Beach, and B. Stillman. 1994. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature* 369:574-578.
 83. Wiehush, L., and C. Hagedorn. 1999. Human cytomegalovirus 86-kilodalton IE2 protein blocks cell cycle progression in G₁. *J. Virol.* 73:9274-9283.
 84. Xiong, Y., G. J. Hannon, H. Zhang, D. Casso, R. Kobayashi, and D. Beach. 1993. p21 is a universal inhibitor of cyclin kinases. *Nature* 366:701-704.
 85. Yu, Z. K., J. L. Gervais, and H. Zhang. 1998. Human CUL-1 associates with the SKP1/SKP2 complex and regulates p21^{CIP1/WAF1} and cyclin D proteins. *Proc. Natl. Acad. Sci. USA* 95:11321-11329.
 86. Zhang, H., G. J. Hannon, and D. Beach. 1994. p21-containing cyclin kinases exist in both active and inactive states. *Genes Dev.* 8:1750-1758.
 87. Zhang, W., R. D. Lane, and R. L. Melgren. 1996. The major calpain isozymes are long-lived proteins. Design of an antisense strategy for calpain depletion in cultured cells. *J. Biol. Chem.* 271:18825-18830.
 88. Zhao, X., J. K. Newcomb, R. M. Posmantur, K. K. Wang, B. R. Pike, and R. L. Hayes. 1998. pH dependency of mu-calpain and m-calpain activity assayed by caspase zymography following traumatic brain injury in the rat. *Neurosci. Lett.* 247:53-57.
 89. Zhao, X., R. Posmantur, A. Kampfl, S. J. Liu, K. K. Wang, J. K. Newcomb, B. R. Pike, K. L. Clifton, and R. L. Hayes. 1998. Subcellular localization and duration of mu-calpain and m-calpain activity after traumatic brain injury in the rat: a caspase zymography study. *J. Cereb. Blood Flow Metab.* 18:161-167.
 90. Zhu, H., J. P. Cong, G. Mamtara, T. Glingens, and T. Shenk. 1998. Cellular gene expression altered by human cytomegalovirus: global monitoring with oligonucleotide arrays. *Proc. Natl. Acad. Sci. USA* 95:14470-14475.
 91. Zimmerman, U. J., M. Wang, J. B. Nelson, F. S. Elkunfite, and L. Lin. 1996. Secretagogue-induced proteolysis of cAMP-dependent protein kinase in intact rat alveolar epithelial type II cells. *Biochim. Biophys. Acta* 1311:117-123.



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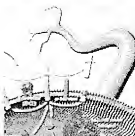
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Ref.: Inomata, M., et al. 1996. *Arch. Biochem. Biophys.* **328**, 129. Mehdi, S. 1991. *Trends Biochem. Sci.* **16**, 150. McGowan, E.B., et al. 1989. *Biochem. Biophys. Res. Commun.* **158**, 432. Tamai, M., et al. 1987. *J. Pharmacobiodyn.* **10**, 678. Komatsu, K., et al. 1986. *Exp. Neurol.* **91**, 23. Tamai, M., et al. 1986. *J. Pharmacobiodyn.* **9**, 672.
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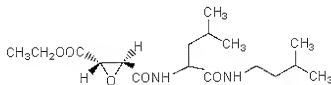
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- * Some inhibitors may permeate some cell systems and not others.
* Inhibitors can be permeable in some cases (particularly with aldehyde-based inhibitors), but may require greater concentrations and longer duration.
* Able to permeate some cells according to several references

CODE	PRODUCT	TARGET	WORKING CONC. GUIDE	SOLUBILITY	CELL PERMEABLE *	REVERSIBLE	QTY	USD
IAR-4148-v	Arphamenine A	Aminopeptidase B	100 - 500 nM	Distilled water		YES	0.5 mg	34
IAR-4149-v	Arphamenine B	Aminopeptidase B	50 - 100 nM	Distilled water		YES	0.5 mg	39
IBS-4093-Pf	Ubenimex (Bestatin)	Aminopeptidase B and Leucine Aminopeptidase	1-10 μ M IC ₅₀ = 4 μ M	Acetone, DMSO	YES	YES	25 mg	142
ILH-4249-v	Leuhistin	Aminopeptidase M	0.1 - 3 μ M K _i = 230 nM (aminopeptidase M)	Distilled water		YES	0.5 mg	36
IAC-4157-v	Actinonin	Aminopeptidase M and Leucyl Aminopeptidase	1 - 200 μ M	Acetic Acid		YES	0.5 mg	34
IPH-4342-v	Phebestin	Aminopeptidase N	K _i = 47 μ M	DMSO			5 mg	100
IAM-4095-v	Amastatin	Aminopeptidase A and Leucyl Aminopeptidase	1 - 10 μ M	DMSO		YES	0.5 mg	69
IDP-4132-v	Diprotin A	Dipeptidyl Aminopeptidase IV	10 - 50 μ M K _i = 2.2 μ M	Distilled water		YES	0.5 mg	18
IEB-4155	Ebelactone A	N-Formylmethionyl Aminopeptidase, Lipase, and Esterase	1 - 10 μ M	MeOH and EtOH	NO*	YES	25 mg	995
IEB-4156	Ebelactone B	N-Formylmethionyl Aminopeptidase, Lipase, and Esterase	1 - 10 μ M	MeOH and EtOH	NO*	YES	25 mg	995
IAB-4009-v	Bradykinin-Potenitiator B	Angiotensin I Converting Enzyme, Peptidyl-dipeptidase A, Kinase II	IC ₅₀ = 1.1 μ M	Distilled water			0.5 mg	30
IAC-4010-v	Bradykinin-Potenitiator C	Angiotensin I Converting Enzyme, Peptidyl-dipeptidase A, Kinase II	IC ₅₀ = 7.1 μ M	Distilled water			0.5 mg	23
IBK-4097-v	Des-Pro ² -Bradykinin	Angiotensin I Converting Enzyme, Peptidyl-dipeptidase A, Kinase II	10 μ M	Distilled water			0.5 mg	30
IFR-4190-v	Foroxymithine	Angiotensin I Converting Enzyme	IC ₅₀ = 12 μ M	Distilled water			0.5 mg	65
IRG-4395-v	KYT-1	Arg-Gingipain	1 - 100 μ M	DMSO			1 mg	263
IKG-4396-v	KYT-36	Arg-Gingipain	1 - 100 μ M	DMSO			1 mg	263
IKK-4374-v	Lys-Lys-Lys-L-Leu-Arg-Arg-Gln-Glu-Ala-Phe-Asp-Ala-Tyr (KKKLLRRQEAFDAY or AIP)	Calmodulin-Dependent Protein Kinase II	IC ₅₀ = 4 nM	Distilled water			0.5 mg	100
IZL-3178-v	Z-Leu-Leu-H (aldehyde) Z-LL-CHO	Calpain	10 - 100 μ M	DMSO	YES	YES	5 mg	40
IES-4096-v	E-64	Calpain, Thiol Proteases	1-10 μ M	Distilled water	NO** (Can Permeate Sf9 Insect Cells)	NO	0.5 mg	37
IEC-4320-v	E-64-c	Calpain and Cathepsin B/H/L, Thiol Proteases	1-10 μ M	DMSO	NO*	NO	5 mg	100

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CODE	PRODUCT	TARGET	WORKING CONC. GUIDE	SOLUBILITY	CELL PERMEABLE*	REVERSIBLE	QTY	USD
IEC-4321-v	E-64-d	Calpain and Cathepsin B/H/L Thiol Proteases	1-10 μ M	DMSO	YES	NO	5 mg	100
ICA-3187-v	Ac-Trp-Glu-His-Asp-H (aldehyde) [Ac-WEHD-CHO]	Caspase-1	10 - 100 μ M	DMSO	NO*	YES	5 mg	200
IAT-3165-v	Ac-Tyr-Val-Ala-Asp-H (aldehyde) [Ac-YVAD-CHO]	Caspase-1/4	10 - 100 μ M	DMSO	NO*	YES	5 mg	195
IAT-3166-v	Ac-Tyr-Val-Lys-Asp-H (aldehyde) Ac-YVKD-CHO	Caspase-1	10 - 100 μ M K _i = 0.038 μ M	DMSO	NO*	YES	5 mg	195
IVD-3204-v	Ac-Val-Asp-Val-Ala-Asp-H (aldehyde) Ac-VDVAD-CHO	Caspase-2	0.0001 - 100 μ M K _i = 3.5 nM	DMSO	NO*	YES	5 mg	300
IAC-3221-v	Ac-Asp-Asn-Leu-Asp-H (aldehyde) Ac-DNLDC-CHO	Caspase-3	1 - 30 nM IC ₅₀ = 3.2 nM	DMSO	NO*	YES	5 mg	212
ICA-3192-v	Ac-Asp-Met-Gln-Asp-H (aldehyde) Ac-DMQD-CHO	Caspase-3	10 - 100 μ M	DMSO	NO**	YES	5 mg	300
IAP-3172-v	Ac-Asp-Glu-Val-Asp-H (aldehyde) Ac-DEVD-CHO	Caspase-3/7/8	1 - 100 μ M	DMSO	NO**	YES	5 mg	195
IBA-3173-v	Biotinyl-Asp-Glu-Val-Asp-H (aldehyde) Biotinyl-DEVD-CHO	Caspase-3/7/8	10 - 100 μ M	DMSO	NO*	YES	1 mg	99
IVA-3182-v	Ac-Val-Glu-Ile-Asp-H (aldehyde) Ac-VEID-CHO	Caspase-6	10 - 100 μ M	DMSO	NO*	YES	5 mg	218
ICA-3194-v	Ac-Asp-Gln-Thr-Asp-H (aldehyde) Ac-DQTD-CHO	Caspase-7/3	50 - 100 μ M	DMSO	NO**	YES	5 mg	250
ICA-3196-v	Ac-Ile-Glu-Thr-Asp-H (aldehyde) Ac-IETD-CHO	Caspase-8/6, Granzyme B	50 - 100 μ M	DMSO	NO*	YES	5 mg	200
ICA-3199-v	Ac-Leu-Glu-His-Asp-H (aldehyde) Ac-LEHD-CHO	Caspase-9	0.01 - 10 μ M	DMSO	NO*	YES	5 mg	250
ICL-3180-v	Ac-Tyr-Val-Ala-Asp-CH ₂ Cl Ac-YVAD-CMK	Caspases	1 - 200 μ M IC ₅₀ = 50 μ M	DMSO	YES	NO	5 mg	200
ICE-3174-v	Z-Asp-CH ₂ -DCB	Caspases	50 - 200 μ M	DMSO	YES	NO	5 mg	195
ICA-3189-v	Z-Glu-Lys(Biotinyl)-Asp-CH ₂ -DMB [Z-EK(bio)D-aomk]	Caspases	1 μ M	DMSO			1 mg	100
ICA-3188-v	Z-Val-Ala-Asp(OMe)-CH ₂ F [Z-VAD(OMe)-FMK]	Caspases	0.05 - 100 μ M	DMSO	YES	NO	1 mg	100
IEC-3644-PI	N-(4-Biphenylacetyl)-Cys(Me)-D-Arg-Phe-N-Phenylethylamide (Cathepsin L inhibitor VI)	Cathepsin L	K _i = 19 nM (Cathepsin L)			YES	5 mg	65
IPA-4397	Pepstatin A (Purity > 90% by HPLC)	Cathepsin D/E, Aspartyl proteases, Pepsin, Renin	1 - 10 μ M	DMSO, Ethanol, or Acetic Acid (9:1 v/v)	NO**	YES (Can Permeate SF9 Insect Cells)	25 mg	75
IAL-3678-PI	Ac-Leu-Leu-Met-H (aldehyde) [ALLM]	Cathepsin B/L, Calpain I/II, Proteasome (weak)	0.1 - 250 μ M	DMSO, MeOH, and EtOH	YES	YES	5 mg	46

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Cell Death in Spinal Cord Injury (SCI) Requires de Novo Protein Synthesis

Calpain Inhibitor E-64-d Provides Neuroprotection in SCI Lesion and Penumbra

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Degradation of cytoskeletal proteins by calpain, a Ca^{2+} -dependent cysteine protease, may promote neuronal apoptosis in the lesion and surrounding areas following spinal cord injury (SCI). Clinically relevant moderate (40 g/cm force) SCI in rats was induced at T12 by a standardized weight-drop method. Internucleosomal DNA fragmentation or apoptosis in the lesion was inhibited by 24-h treatment of SCI rats with cycloheximide (1 mg/kg), indicating a requirement for de novo protein synthesis in this process. To prove an involvement of calpain activity in mediation of apoptosis in SCI, we treated SCI rats with a cell-permeable calpain inhibitor E-64-d (1 mg/kg). Following 24-h treatment, a 5-cm-long spinal cord section centered at the lesion was collected, and divided equally into five segments (1 cm each) to determine calpain activity, as shown by degradation of the 68-kD neurofilament protein (NFP), and apoptosis as indicated by internucleosomal DNA fragmentation. Neurodegeneration propagated from the site of injury to neighboring rostral and caudal regions. Both calpain activity and apoptosis were readily detectable in the lesion, and moderately so in neighboring areas of untreated SCI rats, whereas these were almost undetectable in E-64-d-treated SCI rats, and absent in sham animals. Results indicate that apoptosis in the SCI lesion and penumbra is prominently associated with calpain activity and is inhibited by the calpain inhibitor E-64-d providing neuroprotective benefit.

Key Words: Cell death • Spinal cord injury • Calpain inhibitor • Neuroprotection

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